

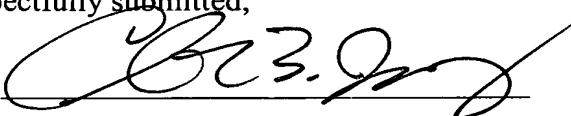
REMARKS

In the Amendment filed by Certificate of Mail on July 24, 2006, Applicants cited the reference Knirel YA et al. "Structural features and structural variability of the lipopolysaccharide of *Yersinia pestis*, the cause of plague". *J. Endotoxin Res.* 2006; 12, 3-9. Applicants enclose herewith a copy of the Knirel et al reference.

Accordingly, it is believed that this Supplemental Response and the Amendment filed by Certificate of Mail on July 24, 2006, represents a complete response to the Examiner's rejections under 35 U.S.C. §§ 103 and 112, first paragraph, and places the present application in condition for allowance. Reconsideration and an early allowance are respectfully requested.

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Structural features and structural variability of the lipopolysaccharide of *Yersinia pestis*, the cause of plague

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Data on the structure and temperature-dependent variations of the lipopolysaccharide (LPS) of *Yersinia pestis* are summarized and compared with data of other enteric bacteria, including other *Yersinia* spp. A correlation between the LPS structure and properties of the LPS and bacterial cultures as well as the LPS biosynthesis control are briefly discussed.

Keywords: Lipopolysaccharide structure, biosynthesis control, antibiotic resistance, core oligosaccharide, lipid A, *Yersinia pestis*

INTRODUCTION

Plague, an acute systemic disease, was the reason of several devastating pandemics resulting in > 200 million human deaths. Although it is presently not a major public health problem, small outbreaks of plague continue to occur throughout the world and at least 2000 cases of plague are reported annually. The causative agent of bubonic and pneumonic plague, the Gram-negative bacterium *Yersinia pestis*, is a category A biothreat agent that could potentially be used as a biological weapon.^{1,2}

As opposed to two other important enteropathogenic *Yersinia* spp., *Y. pseudotuberculosis* and *Y. enterocolitica*, which cause chronic intestinal infections, *Y. pestis* does not depend on a free-living stage. It circulates in natural foci, which involve a rodent reservoir (gerbils, ground squirrels, marmots, voles, pikas, prairie dogs, guinea pigs, rats, etc.) and an insect vector (more than 80 flea species). The high lethality of plague in rodents is necessary for its continued transmission in nature.¹⁻³

Strains of the main subspecies, *Y. pestis* ssp. *pestis*, circulating all over the world and incapable of fermentation of

rhamnose, are usually highly virulent for guinea pigs and humans and were the cause of all plague pandemics.^{1,4} The endemics of the ancient Asian natural plague foci are 'rhamnose-fermentation-positive' *Y. pestis* strains of several Russian non-*pestis* subspecies and Chinese ecotypes (so called pestoides/microtus group/biovar). They are of low virulence or avirulent for guinea pigs and caused only three reported cases of non-lethal human plague that were not accompanied by outbreaks of human-to-human transmission of infection.^{1,4}

The pathogenicity of *Y. pestis* is determined, in part, by a number of virulence determinants that counteract mammalian and insect antimicrobial factors, assuring maintenance of the pathogen in the hosts during the transmission cycle. One of these is lipopolysaccharide (LPS, endotoxin), the major component of the outer membrane of the bacterial cell wall, which mediates cationic-antibiotic- and serum-resistance and infective toxic shock.^{1,2} In contrast to its recent ancestor, the enteric pathogen *Y. pseudotuberculosis*^{5,6} having the full S (smooth)-type LPS, *Y. pestis* possesses an R (rough)-type LPS restricted to an oligosaccharide core and lipid A, while its O-antigen biosynthesis gene cluster is cryptic.⁶ The R-LPS phenotype seems to be beneficial for the bacterium; in particular, the lack of the O-antigen is essential for activation of plasminogen by surface proteases of *Y. pestis*, which plays an important role in the pathogenesis of plague.⁷

Recently, the chemical structure of *Y. pestis* LPS has been elucidated in considerable detail;⁸⁻¹⁵ significant

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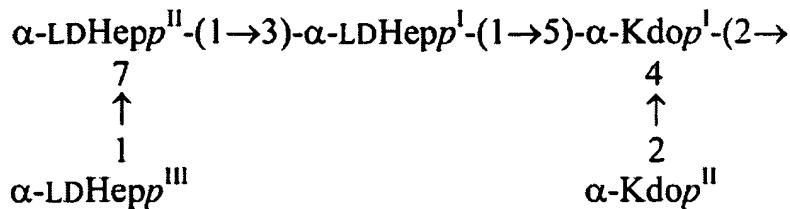


Fig. 1. Structure of the inner core region of the LPS of *Y. pestis*. Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; LD-Hep, L-glycero-D-manno-heptose.

structural variations in the core and lipid A regions have been observed when the bacteria were cultivated at different temperatures.^{10–14} The natural environmental temperatures for *Y. pestis* may vary from 0°C to 42°C, and the temperatures selected for the LPS structure investigations mimicked the conditions in rodents and fleas during winter hibernation (6°C), in active insects (21–28°C) and in awake mammals (37°C). The present review summarizes results of these studies and touches briefly on the problems of structure-to-function relationships and biosynthetic control of the LPS of *Y. pestis*.

LIPOPOLYSACCHARIDE CORE

Typically of enteric bacteria, the LPS core of *Y. pestis* has an inner region of a 3-deoxy- α -D-manno-oct-2-ulosonic acid (Kdo) disaccharide and an L-glycero- α -D-manno-heptose (LD-Hep) trisaccharide (Fig. 1).^{8,9,12} Together with the core of some other genera, such as *Klebsiella*, *Serratia*, *Proteus* and *Providencia*, it belongs to the so-called non-*Salmonella* enterobacterial core type,¹⁶ which is characterized by substitution of LD-Hep^I with β -D-glucose at position 4.^{8,12} The LPS of *Y. pestis* commonly contains glycine that is *O*-linked non-stoichiometrically at an undetermined position in the inner core.¹²

Position 3 of LD-Hep^{II} is occupied by β -D-GlcNAc, which is present in non-stoichiometric amounts in the LPS of all wild-type strains of *Y. pestis* (Fig. 2).^{8,9,12} This component was found also in the R-type (but not S-type) LPS of *Y. enterocolitica*^{17,18} and seems to be a substitute for the missing O-antigen in these bacteria rather than a true core component. Indeed, inactivation in *Y. pestis* of the putative *waaL* gene encoding the ligase for connecting the O-antigen to the core resulted in a mutant that is unable to incorporate GlcNAc into the LPS (authors' unpublished data). The O-antigen gene clusters of *Y. pseudotuberculosis* O:1b and *Y. pestis* showed almost 100% identity between all genes, except for the *wzx* genes, which are only 90.4% identical.⁶ The different genes encode flippase (O-antigen translocase) for translocation through the periplasmic membrane of a pre-assembled undecaprenyl diphosphate-linked O-antigen

repeating unit in the *wzy*-dependent biosynthesis pathway.¹⁹ An altered flippase in *Y. pestis* may thus translocate undecaprenyl diphosphate-linked GlcNAc in the absence of any O-antigen. The ability of flippase to translocate a single GlcNAc residue for further adding to the LPS core has been shown also in *Escherichia coli*.²⁰

When bacteria of the main subspecies *pestis* are cultivated at 37°C, LD-Hep^{III} carries a D-glycero- α -D-manno-heptose (DD-Hep) residue (glycoform A, Fig. 2A) at position 7,¹² a component that is present also in the core of *Y. enterocolitica*.^{17,18} DD-Hep was found in the LPS *Y. pseudotuberculosis* too²¹ but the detailed structure of the core of this bacterium has not been elucidated yet. Moreover, the whole glycoform A core of *Y. pestis* is shared by *Y. enterocolitica*, the latter containing an additional β -D-glucose residue at position 2 of LD-Hep^{II}.^{17,18}

A decrease of growth temperature resulted in two significant changes in the core of *Y. pestis* giving rise to the glycoform B core (Fig. 2B), which differs from glycoform A in: (i) replacement of Kdo^{II} with a D-glycero-D-talo-oct-2-ulosonic acid residue (Ko), i.e. hydroxylation of the 3-deoxy unit in Kdo^{II}; and (ii) replacement of DD-Hep with β -D-galactose, which is attached at the same position on LD-Hep^{III}.^{10,12,13} Almost full replacements were observed at 6°C,¹³ whereas at 25°C the utmost A and B (DD-Hep + Kdo and Gal + Ko) glycoforms co-existed with two mixed DD-Hep + Ko and Gal + Kdo glycoforms.^{8,10,12}

Incorporation of Gal into the core is regulated by the two-component PhoPQ signal transduction system, and a *phoP* mutant of *Y. pestis* grown at 28°C cannot produce the Gal-containing glycoforms whereas the parent strain can.⁹ Taking into account that Gal is present only in bacteria cultivated at lower temperatures,^{10,12,13} it was suggested that, on a temperature decrease, the PhoPQ regulatory system directs the LPS core biosynthesis toward the Gal-containing glycoforms.¹² Therefore, this variation may be important for survival of the bacteria in the flea but the exact role of Gal remains to be determined. Hydroxylation of Kdo, which is out of the control by the PhoPQ system,⁹ may serve for additional stabilization of the outer membrane by hydrogen bonds at lower temperatures.

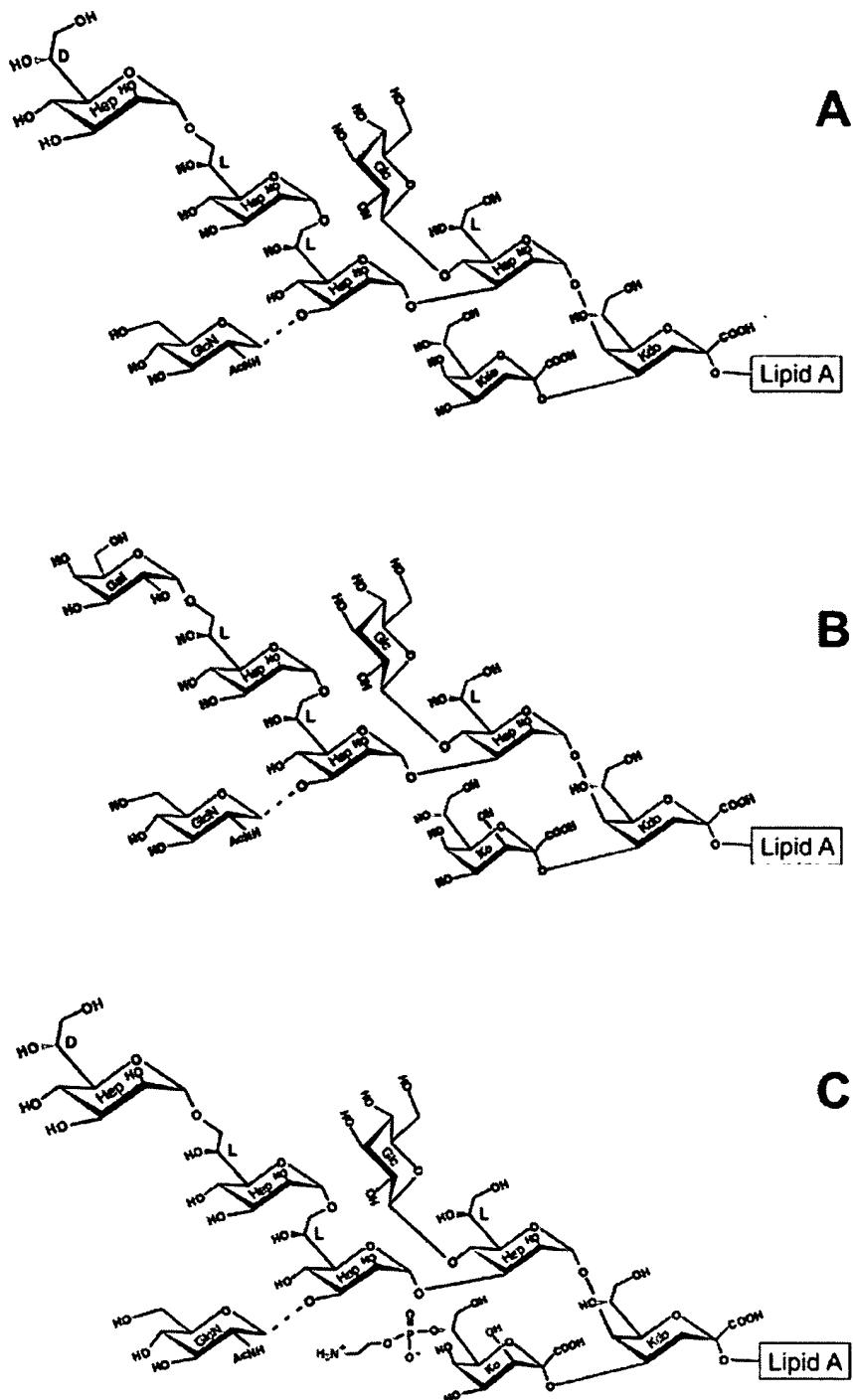


Fig. 2. Structural variants of the LPS core of *Y. pestis* KM218.^{8,10,12,13} (A) the DD-Hep + Kdo core glycoform in bacteria grown at 37°C. (B) the Gal + Ko core glycoform in bacteria grown at 25°C and 6°C. (C) the DD-Hep + EtnPKo core glycoform in bacteria grown at 6°C. At 25°C, glycoform B coexists with glycoform A and two mixed DD-Hep + Ko and Gal + Kdo glycoforms; at 6°C, glycoform C coexists with a minor DD-Hep + EtnPKo glycoform. Glycoform A and B cores contain glycine that is O-linked non-stoichiometrically at an undetermined position. The dotted line indicates non-stoichiometric substitution with GlcNAc. Letters L and D indicate the configuration at C7 of the heptose residues.

A representative of non-*pestis* subspecies, *Y. pestis* ssp. *caucasica*, differs from the main subspecies in the absence of DD-Hep from the core at any temperature¹² and, thus, has a defect in DD-Hep synthesis or transfer. In contrast to strains of the main subspecies, those of ssp. *caucasica* are susceptible to the bactericidal action of nor-

mal human serum.²² However, the lack of DD-Hep from the LPS core is not likely to be responsible for the susceptibility since another DD-Hep-deficient strain of a non-*pestis* subspecies (ssp. *altaica*) is resistant to serum killing.¹ Incorporation of Gal into the core of *Y. pestis* ssp. *caucasica* occurred smoothly at 25°C but poorly at 37°C.¹²

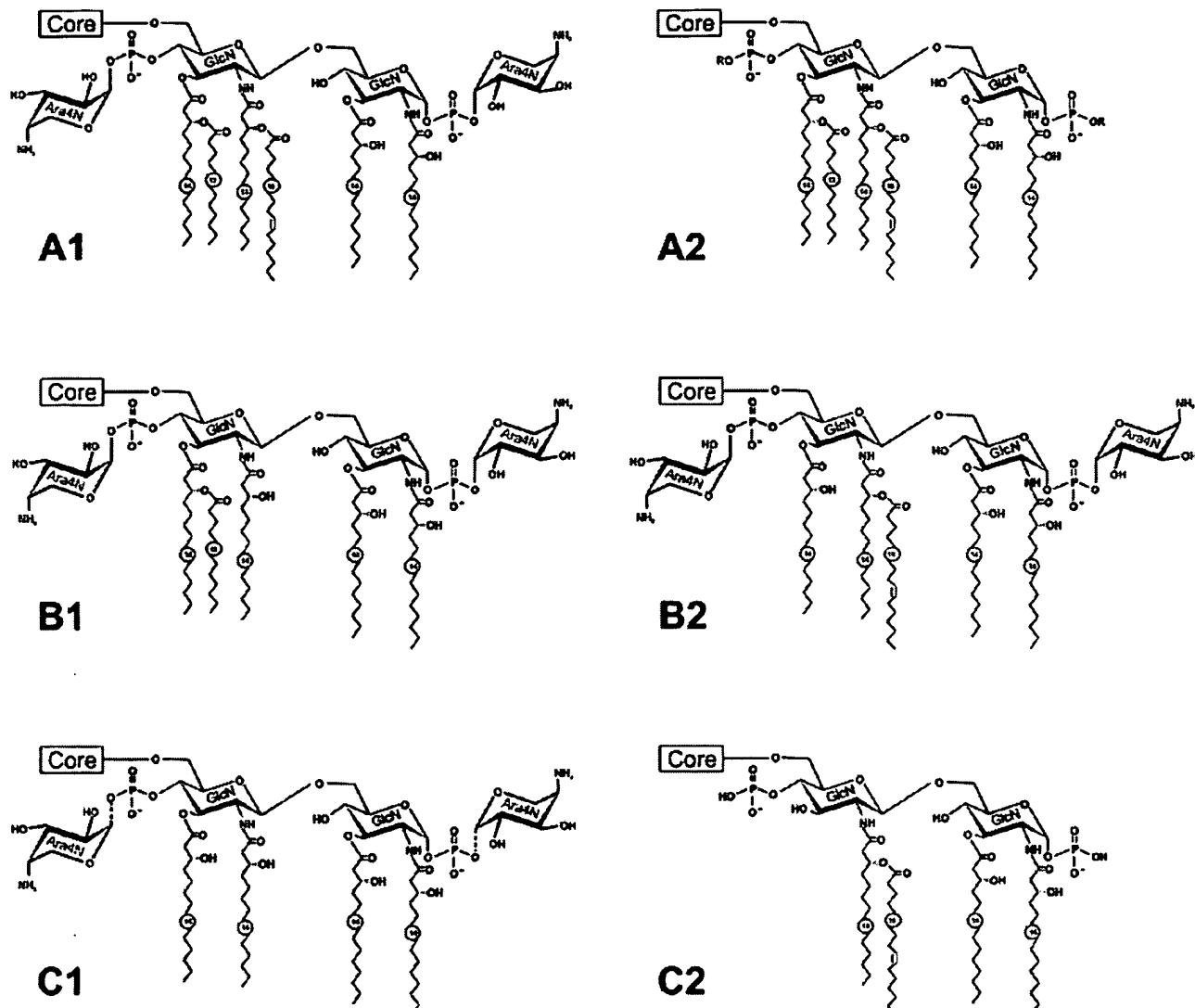


Fig. 3. Structural variants of lipid A of *Y. pestis*.^{11–15} (A) Hexa-acyl lipid A in bacteria grown at 25°C and 6°C associated with the glycoform A and B cores (A1) and at 6°C associated with the glycoform C core (A2). (B) Penta-acyl lipid A in bacteria grown at 25°C (B1) and in an *lpxM* mutant (B2) both associated with the glycoform A and B cores. (C) Tetra-acyl lipid A in bacteria grown at 37°C and 25°C associated with the glycoform A and B cores (C1) and at 6°C associated with the glycoform C core (C2). The dotted line indicates non-stoichiometric substitution with Ara4N.

The contents of the other non-stoichiometric constituents of the core also depend on the growth temperature. Thus, in *Y. pestis* ssp. *pestis* KM218, the content of GlcNAc increased from 0.5 to 0.9 and that of glycine decreased from 0.25 to 0.1 with a temperature growth elevation from 6°C to 37°C.^{12,22}

Remarkably, together with the glycoform B core, at 6°C *Y. pestis* KM218 produces a similar amount of the glycoform C core, which is characterized by the presence of DD-Hep rather than Gal and by attachment of phosphoethanolamine to position 7 of the terminal major Ko and minor Kdo residues (Fig. 2C).¹³ A similar modification of the inner core involving addition of phosphoethanolamine to Kdo^{II} with the aid of a membrane enzyme was induced in *E. coli* by growth with 5 mM CaCl₂.²³ In addition, the glycoform C core lacks glycine

and contains much less GlcNAc than the glycoform A and B cores (0.15 versus 0.5–0.9).

The LPS core of a laboratory deep-rough strain of *Y. pestis*, EV11M, derived from the Russian vaccine strain EV line NIIEG, is restricted to Kdo–Kdo or Ko–Kdo disaccharides.¹² In contrast to wild-type strains, both structural variants are present in similar amounts whether the bacteria are cultivated at 25°C or 37°C.

LIPID A

The general structure of lipid A from *Y. pestis*, as that of the core, is typical of enterobacterial LPS. It has a β-1→6-linked GlcN backbone disaccharide that is phosphorylated at positions 1 and 4'. Four (*R*)-3-hydroxymyristoyl groups

(3HO14:0) are linked as primary fatty acids at positions 2, 2', 3 and 3' of the carbohydrate backbone. In wild-type strains, tetra-acyl, penta-acyl and hexa-acyl lipid A variants have been characterized (Fig. 3).¹¹⁻¹⁵ In addition to four primary acyl groups, hexa-acyl lipid A (types A1 and A2) has one lauroyl (12:0) and one palmitoleoyl (16:1) group linked as secondary fatty acids at positions 3' and 2' of the non-reducing GlcN residue, respectively (Fig. 3A1 and 3A2). In comparison, penta-acyl lipid A (type B1) lacks the 16:1 group (Fig. 3B1). Of two tetra-acyl lipid A variants (types C1 and C2), one possesses only the primary acyl groups (Fig. 3C1) and the other has three 3HO14:0 and one 16:1 groups (Fig. 3C2). An *lpxM* (*waaN, msbB*) mutant of *Y. pestis* has another penta-acyl lipid A (type B2) that lacks the secondary 12:0 group at position 3' but contains the secondary 16:1 group at position 2' (Dentovskaya *et al.*²⁴ and authors' unpublished data) as shown in Fig. 3B2. Hence, *lpxM* is responsible for incorporation of the lauryl residue into lipid A of *Y. pestis*. In *E. coli*, LpxM transfers to the same position the secondary myristoyl group and is able to transfer also the lauryl group.¹⁹

Type A1 hexa-acyl lipid A is produced at lower temperatures but not at 37°C,^{11,12,14} whereas type B1 penta-acyl lipid A accompanies hexa-acyl lipid A at lower temperatures and was also described as a minor variant at 37°C.^{11,14} Modification of all lipid A variants by adding a decanoyl group (10:0) at an undetermined position was reported in *Y. pestis* KIM6+.¹⁴ Type C1 tetra-acyl lipid A is characteristic of bacteria grown at 37°C and is present also at 21–28°C.^{11,12,14} The ratios of differently acylated lipid A variants at 21–28°C may vary from strain to strain and seem to depend significantly on the growth conditions. An alternative type C2 tetra-acyl lipid A is produced at 6°C.¹³ Cold temperature also caused oxygenation of an acyl group (most likely, 16:1) but the resultant modified fatty acid(s) could not be identified.¹³

Interestingly, at 21°C, *Y. pseudotuberculosis* and *Y. enterocolitica* have the same hexa-acyl lipid A as *Y. pestis* but type C2 rather than type C1 tetra-acyl lipid A.¹⁴ At 37°C, both bacteria produce other hexa-acyl lipid A variants having only saturated fatty acids, and *Y. pseudotuberculosis* switches to production of tetra-acyl type C1 lipid A.¹⁴ In *E. coli* at 12°C, the secondary 16:1 group is incorporated at the expense of 12:0 at position 2' by LpxP, which is induced by the cold shock.²⁵ The activity of *lpxP* decreases with a temperature increase and at 30–42°C, LpxL transfers to this position the lauroyl group. In *Y. pestis*, an elevation of growth temperature evidently decreased the activity of *lpxP* too but no secondary saturated acyl group is incorporated instead of 16:1.

A decrease in the number of fatty acid residues at 37°C results in a less immunostimulatory LPS,^{11,14} which may compromise the host's ability to respond rapidly with a regulated and appropriate inflammatory response

to infection. No significant changes in acylation pattern were observed in a *phoP* mutant of *Y. pestis*, which indicates that the PhoPQ regulatory system is not involved in the acylation control.¹⁴

Types A, B and C1 lipid A, but not type C2 lipid A, of *Y. pestis* can be modified by glycosylation of one or two phosphate groups with a cationic sugar, 4-amino-4-deoxy-L-arabinose (Ara4N). In wild-type strains of the main subspecies, the content of Ara4N increases with a temperature decrease^{11,12,14} and is close to stoichiometric at both positions at 25°C.¹² At 6°C, Ara4N-enriched types A1 and C1 co-exist with types A2 and C2 that are completely devoid of Ara4N (Fig. 3).¹³ Remarkably, these Ara4N-deficient lipid A variants and only these variants are associated with the phosphoethanolamine-containing glycoform C core (Fig. 2C).¹³

PhoPQ- and PmrAB-regulated addition of Ara4N to lipid A of *Salmonella enterica* and *E. coli* is known to enhance resistance to polymyxin B and other cationic antimicrobial peptides by a reduction in the affinity of lipid A for these cationic antibiotics.^{19,26} The same regulatory systems are involved in the control of expression of the Ara4N biosynthetic genes in *Y. pestis*,^{14,27} though the regulatory pathway is different.²⁷ Accordingly, a *phoP* mutation in antibiotic-resistant *Y. pestis* strains resulted in sensitivity to polymyxin B,^{9,14,27} cecropin P1^{9,14} and mastoparan,⁹ which could be accounted for by affecting the ability to incorporate Ara4N into lipid A.¹⁴ Polymyxin B resistance of *Y. pestis* ssp. *pestis* correlates with the content of Ara4N in lipid A,^{11,12,14,22} which is higher at 21–27°C than at 37°C^{11,12,14} and increases when the bacteria are grown in the presence of polymyxin B.¹² An appropriate combination of terminal monosaccharides that is achieved in the core at 25°C was also suggested to be important for polymyxin resistance.²² These findings could indicate an adaptive response of *Y. pestis* to growth in fleas, which elaborate antimicrobial peptides as a significant component of their innate immune systems.²⁸ Genetic studies have suggested that *Y. pseudotuberculosis* and *Y. enterocolitica* may use the same regulatory mechanism to control the Ara4N biosynthetic genes²⁷ but, so far, Ara4N has been reported only in lipid A of the former bacterium.¹⁴

In *Y. pestis* ssp. *caucasica* 1146, the content of Ara4N is nearly stoichiometric and not insignificantly influenced by the growth temperature.¹² In spite of that, this strain is about 8-fold more resistant to polymyxin B at 25°C than at 37°C. This increase in resistance may be conferred by a higher content of glycine in the LPS core of *Y. pestis* ssp. *caucasica* grown at 25°C.²²

Lipid A of deep-rough mutant strain EV11M with a truncated LPS core (see above) is characterized by the type C1 tetra-acyl lipid A and a penta-acyl lipid A, which contains an additional 16:0 group at an undetermined position.¹² One or two secondary 16:0 groups are

present in lipid A of *Y. pseudotuberculosis* grown at 37°C.¹⁴ Substitution with Ara4N is partial in lipid A of *Y. pestis* EV11M and its content showed no significant temperature dependence.¹² These data suggest that, in this strain, not only are a number of LPS core and lipid A biosynthesis genes inactivated but also the regulatory system for incorporation of Ara4N is impaired. As one could expect, *Y. pestis* EV11M is highly sensitive to polymyxin B and normal human serum.²²

CONCLUSIONS

Significant progress has been achieved recently in elucidating the chemical structure and structural variations of the LPS of the causative agent of plague, *Y. pestis*. The temperature-dependent variations were found to be accompanied by alterations to LPS bioactivity and resistance of bacteria to antibacterial factors. This suggests a role for the variations in overcoming the defense systems of both warm-blooded mammals (host) and cold-blooded insects (vector), which was confirmed, in part, by biological studies. Both similarity and differences were revealed between the LPS structures of *Y. pestis* and two other important *Yersinia* species, *Y. pseudotuberculosis* and *Y. enterocolitica*, as well as between the LPS structures and properties of *Y. pestis* ssp. *pestis* (main subspecies) and a representative of the non-main subspecies, *Y. pestis* ssp. *caucasica*. A role for the two-component PhoPQ signal transduction system in regulation of both core and lipid A structures in response to environmental conditions was recognized. These findings improve our understanding of pathogen–host interactions at the molecular level and may help formulation of a strategy to overcome the protective mechanisms of *Y. pestis*.

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